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Genetic variation and population structure of the grape powdery mildew fungus, *Erysiphe necator*, in southern France

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Abstract

Erysiphe necator, the causative agent of powdery mildew in grapevine, was introduced into Europe from North America during the middle of the 19th century. Our objective was to analyze the genetic variation and the population structure of the fungus in southern France. The sample comprised 101 isolates and was mainly of flag shoot origin, i.e., infection of sprouting shoots after overwintering of mycelium in buds. RAPD analysis identified different haplotypes that clustered in two genetic groups (A and B). The most frequent haplotypes of each group were found in several different locations in two areas separated by 100 km and throughout the 3 year period. Several haplotypes of both groups originated from flag shoots and were recovered over successive years indicating that there is no correlation between genetic group and overwintering mode. All isolates of group A were of mating type +, but those in group B could be either + or −. Lower genotypic diversity was detected within group A than within group B. These results were consistent with the hypothesis that group A reproduces only asexually.

Introduction

Powdery mildew, caused by *Erysiphe necator* (formally *Uncinula necator*) (Braun and Takamatsu, 2000), is one of the major disease of grapevine throughout the world (Bulit and Lafon, 1978; Pearson, 1988). The fungus was introduced into Europe from the eastern and central United States during the middle of the 19th century (Viala, 1885). *Erysiphe necator* is a haploid, biotrophic ascomycete, it can overwinter as hyphae within buds (Sall and Wrysinski, 1982; Pearson and Gärtel, 1985) or as cleistothecia that contain the sexual spores (Pearson and Gadoury, 1987; Cortesi et al., 1995). Since the fungus is heterothallic (Smith, 1970), the fruiting bodies form only when mycelia of both mating types are present on infected tissues. Infections originating from perennation in buds result in developing shoots covered by mycelium and conidia shortly after bud break. These infected shoots, called flag shoots, produce large quantities of asexual inoculum early in the growing season. After the establishment of the disease, either from mycelia in the bud or from ascospores, several asexual cycles occur by means of conidia that infect green tissues during the growing season.

Genetically distinct but morphologically similar groups have been identified in populations of *E. necator* (Délye et al., 1997; Evans et al., 1997;

Délye and Corio-Costet, 1998; Stummer et al., 2000; Miazzi et al., 2003). Délye et al. (1997) distinguished a group of isolates in Europe corresponding to flag shoots, and another group comprising foliar isolates collected later in the growing season. When they paired the flag shoot isolates with isolates from the other group, only sterile cleistothecia were obtained. In addition, sexual reproduction within the group from flag shoots appeared to be impossible because its members were of the same mating type. The authors then proposed the existence of two biotypes, a putative asexual biotype associated with flag shoots and a biotype capable of sexual reproduction.

These deductions were based on small samples from populations mainly taken in France and Germany and were not fully corroborated by further analyses performed in other regions. Two genetic groups also have been observed in Australia but without reference to flag shoots (Evans et al., 1997; Stummer et al., 2000). These groups were shown to correspond to those identified in Europe (Délye et al., 1999; Stummer et al., 2000). The group corresponding to the putative asexual biotype also presented only one mating type in contrast to the other group that showed the two mating types. The reasons given in Europe to explain the lack of recombination (Délye et al., 1997) did not appear to be supported in Australia since (i) an isolate of the putative asexual group was recovered throughout the growing season indicating no ecological difference between groups (Stummer et al., 2000) and (ii) viable progenies have been obtained through controlled crosses in the laboratory (Stummer et al., 2000; Stummer and Scott, 2004).

In a large sample size collected from different locations in southern Italy, Miazzi et al. (2003) also identified several phenetic groups. One of the groups comprised all the isolates obtained from flag shoots and a control isolate belonging to the putative asexual group of Délye et al. (1997). However, this group comprised the two mating types and no sexual incompatibility was detected with the other group. Miazzi et al. (2003) thus retained the assumption that a temporal separation within the growing season prevented sexual recombination between groups. Cortesi et al. (2004) intensively sampled isolates from flag shoots in one vineyard over a period of 5 years. They did not mention whether or not genetic analysis identified different groups but also found two mating types within this flag shoot population.

A variation in the population structure depending on the region may explain the discrepancies in the literature. However, some experimental reasons, such as differences in the sampling scheme, sample size and marker system, could also explain these discrepancies. Whatever the reasons, it would be useful to carefully analyze the structure of additional samples of *E. necator*. The main objective of this study was, therefore, to analyze the group composition and genotypic diversity in a *E. necator* sample from southern France in relation to the presence of flag shoots. The working hypothesis tested was that flag shoot isolates correspond to a unique genetic group and have only one mating type. The data obtained should contribute to resolve the conflict between previously reported results and could be used to develop a different basis for further research on the population biology of this important pathogen of grape.

Materials and methods

Sampling of Erysiphe necator

We undertook this study in a part of southern France that has several interesting characteristics with respect to the epidemiology of powdery mildew: (i) the climate is very favourable to the disease, (ii) abandoned vineyards are common; they do not receive chemical control and may be a source of primary inoculum, and (iii) cv. Carignan is widespread and one of the most favourable cultivars for flag shoot infection. To explore the genetic structure of the fungus within this region we sampled isolates across several spatial scales and over several years (Table 1). Isolates were obtained from two areas, from several vineyards (sites) within each area and from several different vines in some sites. The two areas were located in different districts, Hérault and Ardèche, and were separated by about 100 km. The longest geographical distance between sites was approximately 10 and 3 km in the first and in the second area, respectively. A total of 100 isolates was obtained, 79 in the first area and 21 in the second. Seventy-two isolates were recovered from flag shoots and the remaining either from leaves or

Table 1. Origin, date of sampling and genetic characterization of *Erysiphe necator* isolates sampled in southern France

Site	District	Town	Tissues	Cultivar	Date of sampling	Genetic group	RAPD haplotype (No. of isolates)
1	Hérault	Assas	Flag shoots	Carignan	April 1999	A	1 (7), 4 (2), 6 (1)
		Assas	Flag shoots	Carignan	April 2000	A	1 (13), 2 (2), 3 (2)
2		Montpellier	Flag shoots	Carignan	April 1999	A	1 (2)
		Montpellier	Leaf	Carignan	April 1999	B	20 (1)
		Montpellier	Flag shoots	Carignan	April 2000	A	1 (10)
3		Montpellier	Flag shoots	Carignan	April 1999	A,B	1 (7), 7 (3), 8 (1), 9 (1)
		Montpellier	Flag shoots	Carignan	April 2000	A,B	1 (10), 7 (1), 8 (1), 9 (1), 16 (1)
4		Mauguio	Flag shoots	Carignan	April 1999	B	8 (4), 9 (1)
5		Montpellier	Leaf	Syrah	October 1999	B	9 (1)
		Montpellier	Leaves	Several	July 2000	B	7 (1), 8 (1), 13 (1), 14 (1), 15 (1)
		Montpellier	Leaves	Carignan	May 2001	B	7 (2)
6	Ardèche	Faugères	Berry	Jacquez	July 2000	B	7 (1)
		Faugères	Leaf	Carignan	July 2000	A	2 (1)
7		Faugères	Berry	Jacquez	July 2000	B	7 (1)
		Faugères	Leaf	Cinsault	July 2000	A	1 (1)
8		Faugères	Leaf	Aramon	July 2000	B	19 (1)
9		Faugères	Leaves	Villard Noir	July 2000	A	1 (2), 2 (1)
		Faugères	Leaf	nd[a]	July 2000	A	1 (1)
10		Faugères	Leaf	Carignan	October 2000	B	10 (1)
11		Faugères	Leaf	Gamay	October 2000	B	18 (1)
12		Faugères	Flag shoot	Carignan	May 2001	A	1 (1)
13		Payzac	Flag shoot	Carignan	May 2001	A	5 (1)
14		Faugères	Leaf	nd	September 2001	B	17 (1)
14		Faugères	Leaves	nd	September 2001	B	7 (1), 10 (1)
15		Faugères	Leaf	nd	September 2001	B	7 (1)
17		Payzac	Leaves	nd	September 2001	B	10 (1), 12 (1)
18		Payzac	Leaf	Gamay	November 2001	B	7 (1)
19		Payzac	Leaf	Villard Noir	November 2001	A	1 (1)

[a]Not determined.

The isolate collected in 1997 in the greenhouse at Montpellier, used as a standard in the RAPD analyses, corresponds to haplotype 11 of group B and does not appear in this table.

berries. We added to this sample an isolate obtained in 1997 from a plant growing in the greenhouse, which was used as a standard throughout the RAPD analysis.

Plant material

Two *Vitis vinifera* cultivars were used for this study. Cultivar Cinsault was used as *in vitro* plantlets, obtained and cultured according to Péros et al. (1998), to provide leaves for single-spore isolation and the maintenance of *E. necator* isolates. Cultivar Cabernet-Sauvignon was used as detached leaves from plants grown in the greenhouse. One-bud cuttings were prepared in spring from 1-year-old branches collected during the preceding winter and stored at 4 °C. Cuttings were established at high density in flat boxes filled with vermiculite. They were placed in a growth chamber at 22–25 °C with a 12 h cycle of light and dark. After 6–8 weeks, rooted cuttings were transferred into 2 l pots containing a mix of soil and vermiculite (2:1), fertilized (Osmocote, Scotts Europe B.V., Heerlen, The Netherlands) and grown in the greenhouse. Plants were trimmed during spring and summer to allow the development of new shoots with young glossy leaves suitable for inoculation.

The second expanded youngest leaves of greenhouse-grown plants were collected, washed in tap water and disinfected in a bleach solution (0.24% active chloride) for 1 min 30 s. After three rinses in sterile distilled water, the leaves were dried between several layers of sterile paper. The petiole was then reduced to a size of 1.5–2 cm. Leaves were placed upper surface up on 8 g l^{-1} agar medium in 9 cm diam Petri dishes, the remaining part of the petiole being inserted into the medium. For the *in vitro* material, when plantlets had 8–10 nodes, the five uppermost leaves of the same plantlet were cultured in one plate using the same

procedures as for the leaves from potted plants but without disinfection.

Single-spore isolates

Within 2 days after collection from the vineyard, infected tissues were brushed onto detached leaves of cv. Cabernet-Sauvignon. The inoculated leaves were incubated for 10–15 days at 25 °C under 16 h d^{-1} of illumination (40 μE m^{-2} s^{-1}). Either a single or a few conidia from the same conidial chain were then removed using a glass needle under the binocular microscope. Conidia were spotted onto newly detached leaves from the greenhouse or onto leaves from *in vitro* plantlets of cv. Cinsault. The process was repeated once. The clones were maintained on leaves from *in vitro* plantlets and subcultured on fresh leaves every 4–5 weeks. All inoculated material was cultured under the temperature and light regimes described above.

DNA extraction

The mass production of conidia of each isolate was performed on detached leaves of cultivar Cabernet-Sauvignon. The leaves were inoculated by brushing infected tissues and incubated for 15 days. Leaves were then carefully checked under the binocular microscope and those having contaminating fungi were discarded. Conidia were harvested using a cyclone separator connected to a vacuum pump as described by Evans et al., (1996). Conidia were freeze-dried and DNA was extracted using the CTAB method described by Rogers and Benedich (1985), modified for use with the fungus *Eutypa lata* (Péros et al., 1996). For use with *E. necator*, 400 mg of glass beads (1.8 mm diam) were added to the tube containing the freeze-dried conidia and 500 μl volume of extraction buffer. The tube was vortexed for 1 min before incubation at 65 °C for 30 min. The DNA was quantified on 0.8% agarose gels stained with ethidium bromide by visual comparison with different quantities of lambda DNA (Invitrogen, Cergy-Pontoise, France).

RAPD amplifications

Each reaction volume of 25 μl included 1.5 units of Taq DNA polymerase (QBiogen, Illkirch, France), 1×buffer [10 mM Tris-HCL pH 9, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mg ml^{-1} BSA] provided with the enzyme, dNTP at 120 μM, 30 ng of primer and approximately 2 ng of template DNA. The reaction was overlaid with a drop of mineral oil. A thermal cycler (PTC100, MJResearch, Watertown, MA, USA) was programmed for an initial step of 4 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 38 °C, 1 min at 72 °C, and a final step of 6 min at 72 °C. Amplified products were analyzed by electrophoresis (TBE 0.5X) in 1.6% agarose gels at 5 V per cm for 4 h and a molecular size marker (1-kb ladder, Invitrogen) was included on each gel. Fragments were detected by staining with ethidium bromide and gels were photographed under UV light.

Sixty-three decamer primers (Operon, Alameda, USA) were screened to identify those that gave well-amplified, polymorphic and reproducible bands. Some were chosen because they had already revealed polymorphism in *E. necator* (Délye, 1997) or in *Eutypa lata* (Péros and Berger, 1999). The reproducibility of patterns was evaluated using different DNA preparations from a few representative isolates. Finally, seven primers were used to compare all isolates. These primers were A16 (5′-AGCCAGCGAA-3′), C08 (5′-TGG ACCGGTG-3′), E07 (5′-AGATGCAGCC-3′), J20 (5′-AAGCGGCCTC-3′), P06 (5′-GTGGGC TGAC-3′), P14 (5′-CCAGCCGAAC-3′), U19 (5′-GTCAGTGCGG-3′). Each reaction was repeated at least twice for each isolate. A negative control reaction with no DNA template and a positive control reaction with DNA from a standard isolate were included in each run. In addition, DNA templates representative of the two genetic groups were included in each run when experiments were replicated. Only data that were identical in all runs were considered. All these precautions were taken because important problems associated with multilocus marker systems like RAPD are the variability between PCR runs and faint bands (Pérez et al., 1998).

Polymorphic bands were scored as separate putative loci with two alleles. The presence and absence of the amplification product corresponded to the positive and null allele, respectively. The scoring served to define the multilocus genotype (haplotype) of each isolate. The genetic dissimilarity (D) between all pairs of haplotypes was calculated using the coefficient of similarity given by Jaccard (1908): $S = a/(a+b+c)$ and $D = 1-S$,

where a is the number of fragments shared by the two haplotypes, and b and c are the number of fragments observed in each haplotype. The programme CLUSTER developed by J. Brzustowski (*http://www.biology.ualberta.ca/jbrzusto*) was used to perform distance calculation in pair-wise comparisons and to establish relationships between haplotypes using the unweighted pair group method of arithmetic averages (UPGMA). CLUSTER was also used to perform a stability analysis with 1000 bootstrap resamplings. An unrooted tree was then plotted using the programme DRAWTREE of PHYLIP software (Version 3.6, J. Felsenstein, Departement of Genetics, University of Washington). To analyze the genotypic diversity within our sample, we followed the suggestions of Grünwald et al. (2003). We calculated the index of richness $E(g_n)$ using the rarefaction method and the index of evenness $E_5 = (N_2 - 1)/(N_1 - 1)$ where N_1 and N_2 are Hill's numbers (Hill, 1973). These calculations were performed with R software (*http://www.r-project.org/*) with functions of the Vegan R package. The function *rarefy* gave the index S ($= E(g_n)$) and its standard error and the function *renyi* gave the Hill's numbers for scales 1 and 2.

Determination of mating type

Délye (1997) reported that amplification with primer E07 generated a fragment of around 1000 bp that was present for isolates of mating type + and absent for isolates of mating type – in a sample of 90 isolates from both groups and diverse geographic regions. Furthermore, the analysis of 32 individuals from the same progeny showed that the marker always co-segregated with mating type. The marker was therefore used to assess the mating type of each isolate but was not used in the genetic diversity analysis.

Nested allele-specific PCR (NAS-PCR)

We performed with slight modifications the NAS-PCR assays developed by Délye et al. (1999) to identify three nucleotide differences between the two genetic groups of *E. necator* for the gene encoding eburicol 14α-demethylase (*CYP51*). Briefly, a first-round PCR was used to amplify a fragment of 1756 bp within the *CYP51* sequence using primers C14 and C14R. A 1 µl aliquot of the first-round PCR product was then used in the second amplification using each of the six specific primer pairs. Each reaction volume of 25 µl included 0.2 units of Taq polymerase (Qbiogene) and primers were used at a final concentration of 0.1 µM each. Amplification products were run on 1.6% agarose gels (TBE 0.5X) at 100 V for 2–3 h.

PCR amplification and sequencing of ITS regions

The rDNA-ITS regions were amplified using the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990). Each reaction volume of 50 µl included 0.4 units of Taq DNA polymerase (Qbiogen), 1×buffer (10 mM Tris-HCL, pH 9, 50 mM KCl, $MgCl_2$ 1.5 mM, 0.1% tritonX100, 0.2 mg ml^{-1} BSA) provided with the enzyme, 200 µM of dNTP, 0.8 µM of each primer and approximately 2 ng of template DNA. The thermal cycler was programmed for 37 cycles of 0.5 min at 94 °C, 1 min at 52 °C, and 1.5 min at 72 °C. Sequences from both strands were supplied by Genome Express (Meylan, France). Sequences were aligned by using ClustalW (Thompson et al., 1994) at *http://www.infobiogen.fr* along with the four ITS sequences of *E. necator* available in GenBank: AF011325 from California; AF073346, from Australia; and AF049331 and AF0449332 that are representative of both genetic groups identified in Europe.

Results

The seven primers generated 20 polymorphic fragments that were easily scored and very reproducible. Nineteen were used to compare the genetic relatedness of 101 *E. necator* isolates. Isolates corresponded to a total of 20 haplotypes that clustered in two different groups (A and B). The average Jaccard's similarity was 9% between groups, 76% within group A and 63% within group B. Three bands were found only in group A and two only in group B; three of these specific markers were obtained with primer U19 (Figure 1). A combination of the UPGMA algorithm applied to the dissimilarity matrix of haplotypes and bootstrap analysis indicated that the two genetic groups were very distinct

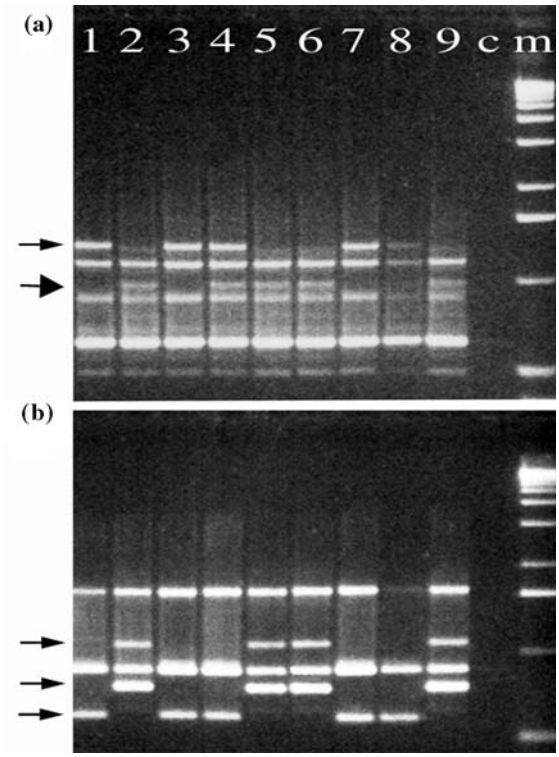

Figure 1. Examples of RAPD patterns generated by primer E07 (a) and primer U19 (b) revealing the genetic diversity between groups in *Erysiphe necator*. Arrows of small size show four markers used in the diversity analysis, the large arrow shows the marker used to determine mating type. Group A isolates: lanes 2, 5, 6 and 9; group B isolates: lanes 1, 3, 4, 7 and 8; lane c: control with no DNA template; lane m: molecular weight marker (kb-ladder).

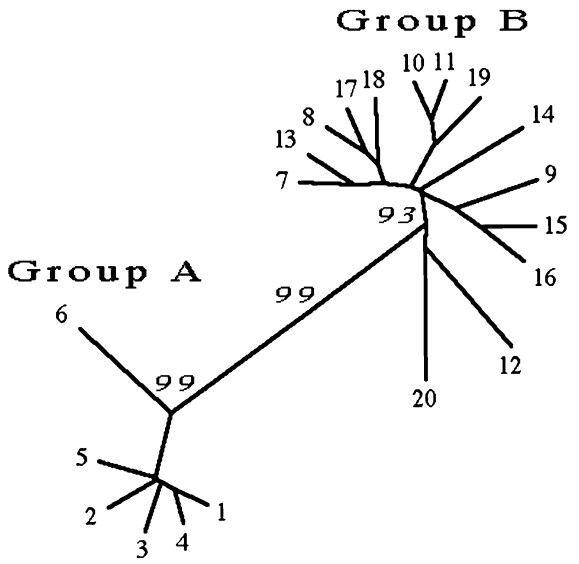

Figure 2. UPGMA tree showing genetic relationships between twenty haplotypes detected by 19 RAPD markers among 101 *Erysiphe necator* isolates collected in southern France based on pairwise comparisons using the Jaccard coefficient. Only bootstrap values ≥90% are indicated.

genetic entities (Figure 2). Group A comprised 65 isolates that corresponded to six haplotypes, the most frequent haplotype (no. 1) being represented by 55 isolates, and group B comprised 36 isolates separated in 14 haplotypes, 10 haplotypes comprising a unique isolate (Table 2). Within each group only two bootstrap values of the clustering analysis were above 90%: haplotype 6 was distinguished within group A and haplotypes 12 and 20 constituted a separate sub-group within group B (Figure 2). The smallest sample size ($n = 36$) was used to calculate richness using the rarefaction method. Group A ($S = 4.62 \pm 0.90$) was much less rich than group B ($S = 13.72 \pm 0.45$). Evenness was also lower in group A ($E5 = 0.41$) than in group B ($E5 = 0.66$).

The 72 isolates originating from flag shoots were distributed in the two groups: 56 isolates in group A and 14 in group B (Table 2). Four haplotypes of group B comprised isolates from flag shoots. The most frequent haplotype of group B (no. 7) was recovered from flag shoots, leaves and berries (Table 1).

The RAPD patterns for all isolates of group A showed the marker E07-1000, whereas this marker was either absent or present in the patterns of isolates of group B (Figure 1). This indicates that all isolates of group A were of the mating type + and that group B included isolates of both mating types. The ratio for the two mating types within group B was 17:19, not significantly different from 1:1 ($\chi^2 = 0.11$, $P = 0.74$). In addition, there was no variation in mating type among isolates of the same RAPD haplotype in group B. This suggests that RAPD haplotypes represent clonal lineages.

The most frequent haplotypes of group A (no. 1) and of group B (no. 7) were obtained from several sites in the two districts and each year during the 3 year sampling period (Table 1). The second most frequent haplotype of group A was recovered in 2000 in both districts. Two other frequent haplotypes of group B (no. 8 and 9) were sampled in several sites in the Hérault district and in two successive years. Haplotype no. 10 was obtained from three sites in the Ardèche district and in two successive years. The recovery of the same RAPD

Table 2. Characterization of haplotypes in a *Erysiphe necator* sample from southern France based on RAPD analysis, NAS-PCR and rDNA sequencing

RAPD haplotype	Group[a]	No isolates Total (Flag shoots)	Mating type[b]	NAS-PCR[c]				ITS1 position 60
				1	2	3	4	
1	A	55 (49)	+	–	–	+	–	T
2	A	4 (2)	+	–	–	+	–	T
3	A	2 (2)	+	–	–	+	–	T
4	A	2 (2)	+	–	–	+	–	T
5	A	1 (1)	+	–	–	+	–	T
6	A	1 (1)	+	–	–	+	–	T
7	B	11 (3)	–	+	+	–	+	C
8	B	7 (6)	+	+	+	–	+	C
9	B	5 (4)	–	+	+	–	+	C
10	B	3 (0)	+	+	+	–	+	C
11	B	1 (0)	+	+	+	–	+	C
12	B	1 (0)	–	+	+	–	+	C
13	B	1 (0)	–	+	+	–	+	C
14	B	1 (0)	+	+	+	–	+	C
15	B	1 (0)	+	na	na	na	na	C
16	B	1 (1)	+	+	+	–	+	C
17	B	1 (0)	+	na	na	na	na	C
18	B	1 (0)	+	nt	nt	–	+	C
19	B	1 (0)	+	nt	nt	–	+	C
20	B	1 (0)	–	nt	nt	–	+	C

[a]Assignment in genetic groups was based on the cluster analysis of genetic distance in pairwise comparison between haplotypes (see Figure 2).

[b]Determination of mating types was based on the presence or absence of a RAPD fragment of around 1000 bp amplified by primer E07 (see Figure 1).

[c]Nested allele specific PCR (NAS-PCR) was performed with the method used to distinguish genetic groups I and III according to Délye et al. (1999); 1: 125-bp fragment amplified (+) or not (–) with primer pair MUT2(III)/UD1DM in group III isolates; 2: 447-bp fragment amplified (+) or not (–) with primer pair MUT3(III)/M1I in group III isolates; 3: 630-bp fragment amplified (+) or not (–) with primer pair MUT4(I–II)/M1 in group I isolates; and 4: 630-bp fragment amplified (+) or not (–) with primer pair MUT4(III)/M1 in group III isolates; na: no amplication in the first-round PCR with primer pair C14/C14R; nt: not tested.

haplotypes of group B in successive years, as the finding of group B isolates in flag shoots, strongly supports the conclusion that some individuals of group B are able to overwinter in buds.

Nested-PCR in the *CYP51* gene was undertaken on selected isolates representing the twenty different haplotypes (Table 2). In our conditions, the first-round PCR did not give the expected product for two haplotypes despite several attempts with different isolates. In addition, two of the six primer pairs designed for the second-round PCR did not work. Three primer pairs gave the fragments expected for group III of Délye et al. (1999) with haplotypes from group B but not with haplotypes from group A. The last primer pair gave the fragment expected for group I of Délye et al. (1999) with haplotypes from group A but not with haplotypes from group B.

Forward and reverse rDNA sequences were obtained for the twenty haplotypes. The alignment of all sequences revealed a clear polymorphism at position 60 in ITS1 where group A and group B isolates had a C and T nucleotide, respectively (Tables 2 and 3). Only three reverse sequences were too short to confirm data obtained with the forward sequences. Four base substitutions concerning different haplotypes were observed at the beginning of the sequence but were not confirmed on both strands. The nucleotide length for ITS1, 5.8S and ITS2 was 224, 154 and 188, respectively. We then performed an alignment with the four sequences already deposited in the GenBank database (Table 3). The base substitution at position 60 also differentiated the groups I (AF049332) and III (AF049331) defined by Délye et al. (1999). However, seven indels, one inversion and two substitutions distinguished the latter sequences from those obtained for groups A and B. The sequence of the Californian isolate (AF011325) had two substitutions and one deletion compared with

Table 3. Observed variable sites in the internal transcribed spacer sequences (rDNA-ITS) obtained in this study for genetic groups A and B of *Erysiphe necator* and comparison with the sequences previously deposited in the GenBank database for this fungus

Position (bp)[ab]	ITS1												ITS2		
	16–17	19	26	28	30–31	60	89	102	108	122	133	142–143	471	491	492
Group A[b]	AG	C	T	–	TC	**T**	C	C	G	G	C	GC	G	G	G
Group B	AG	C	T	–	TC	**C**	C	C	G	G	C	GC	G	G	G
AF049332	AG	–	T	T	CT	T	–	–	–	–	–	CG	G	C	C
AF049331	AG	–	T	T	CT	C	–	–	–	–	–	CG	G	C	C
AF073346	AG	C	T	–	TC	C	C	C	G	G	C	GC	C	G	G
AF011325	CT	C	–	–	TC	C	C	C	G	G	C	GC	G	G	G

[a]Only variable positions are shown and these are numbered from the beginning of ITS1.
There is only one variable location at position 60 between the sequences of group A and group B (in bold).
[b]Sequence for haplotype 1 (isolate CO02) was deposited in GenBank, accession n°DQ189089.

our rDNA sequence data. The ITS1 sequence obtained in Australia (AF073346) was identical to our sequence for group B but had at position 471 in ITS2, a C nucleotide whereas all other sequences had a G nucleotide.

Discussion

Our results showed that *E. necator* was differentiated into two major genetic groups within the region of sampling and established a link between the groups we defined as groups A and B and those already described in Europe (Délye et al., 1997) and Australia (Stummer et al., 2000). Despite different sample sizes and marker systems, there is a good agreement between our data and those obtained in four European countries and in Australia. We confirmed that (i) group A comprised isolates with only the mating type +, (ii) group A showed a lower genotypic diversity than group B and (iii) the genetic difference between groups was larger than the genetic difference within groups. These results are consistent with the hypotheses that group A reproduces only asexually and that the two groups do not recombine efficiently in natural conditions. The greater genotypic diversity within group B compared to group A may be explained by the possibility for recombination within group B, whereas new genotypes within group A may only appear after mutation.

A different picture emerged in Italian populations that showed a maximum or a very high level of genotypic diversity (Miazzi et al., 2003; Cortesi et al., 2004). In addition, when groups were distinguished, the putative group A contained the two mating types and the genetic similarity between groups was not clearly distinguishable from the values observed within groups (Miazzi et al., 2003). A difference in the number of markers assayed cannot explain the great diversity observed in Italy. For instance, Miazzi et al. (2003) found that all 291 isolates of group B were different RAPD haplotypes using 55 polymorphic fragments but Délye et al. (1997) identified 38 RAPD haplotypes among 53 isolates of group B using around 150 polymorphic fragments. Greater numbers of haplotypes would be expected using more markers. A more possible explanation is that populations of *E. necator* have a different genetic structure in Italy compared to other European countries and Australia. For instance, group A could possess two mating types that were both present at high frequency in Italy but not elsewhere. Furthermore, isolates taken from flag shoots within each year in Italy corresponded each to a different genotype that very rarely occurred in successive years (Cortesi et al., 2004). The authors thus suggested that some of these shoots were infected by ascospores released early in the season and not by the overwintering mycelium. The early discharge of ascospores has been detected in other regions (Gadoury and Pearson, 1990; Jailloux et al., 1999) but could be more frequent in Italian vineyards.

One important finding in our study is that both genetic groups can overwinter in buds. The proposal by Délye et al. (1997) that group A is associated with flag shoots was based only on a very small number of isolates. Our results, based on a larger number of isolates as well as of vineyards surveyed, clearly do not confirm this

assumption. The survival of clones from year to year in buds and the existence of a 1:1 ratio for mating types within group B indicated that this group has a mixture of clonal and sexual reproduction in our region of sampling. We also obtained an indication that population structure varied depending on the vineyard. For instance, for a similar number of flag shoots sampled (27 vs. 26), all isolates from site 1 were from group A whereas those from site 3 represented the two groups. The most frequent haplotypes of both groups were identified in the two districts indicating that past or current movement of genotypes probably occurred at the geographical scale of our study (100 km). Examples of genotypes recovered over longer distances (250–1000 km) have been described for group B in France and India (Délye et al., 1997) and for both groups in Australia (Stummer et al., 2000).

The rDNA analyses showed that the two genetic groups differed only at position 60 in ITS1 with a nucleotide T for group A and nucleotide C for group B. This confirms the correspondence between our groups A and B and those described by Delye et al. (1999), as well as the absence of polymorphism within each group at that position. However, the alignment of available ITS sequences revealed some variation within *E. necator*. As we sequenced both strands of DNA for a total of twenty isolates, we are therefore confident in our data. In contrast, the other sequences previously deposited in the GenBank database corresponded to only one isolate. The analysis of other isolates from the regions where these isolates were sampled appears necessary before making conclusions about the polymorphism observed at other positions than position 60 in ITS1. Based on the polymorphism at that position, both the Californian and Australian isolates could be tentatively placed within group B.

In France, the absence of within-group polymorphism for the three available sequences, i.e., rDNA (this study, Délye et al., 1999), *CYP51* gene (Délye et al., 1999) and β-tubulin gene (AY074934, deposited in GenBank by Amrani and Corio-Costet, 2002), was expected for a recently introduced pathogen. Assuming that the two genetic groups originated in small and separate founder populations, they may have undergone a severe bottleneck. One would therefore expect the diversity found in *E. necator* populations in Europe and Australia to be lower than the diversity existing in North America. Assessment of the diversity of *E. necator* in North America appears to be a prerequisite to gain more insight into the evolution of this species. Such a study would help to determine whether or not more than two genetically isolated groups exist within the native area.

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